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Prevalence Patterns and Genotypes of GB Virus C/Hepatitis G Virus Among Imprisoned Intravenous Drug Users

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An RT-PCR assay using primers from the 5′-UTR of the GBV-C/HGV genome was used to detect viremia, and a serological assay was used to detect past exposure to GBV-C/HGV, in sera from 106 imprisoned Greek intravenous drug users. High seroprevalence rates indicative of the parenteral route of transmission of the virus were found (32.1% for GBV-C RNA and 46.2% for anti-GBV-C E2). These rates were nonetheless lower in comparison to the corresponding rates of HCV infection markers (64.2% for HCV RNA and 77.4% for anti-HCV). Statistically significant univariate associations were observed between GBV-C-RNA positivity and younger age (P = 0.006) and HCV-RNA positivity (P = 0.024), as well as with higher serum alanine aminotransferase levels (P < 0.001); this latter association was shown to be independent of coinfection with HCV and of age by a multiple logistic regression model. Apparently, GBV-C/HGV had spread readily by needle-sharing in prison, while causing acute subclinical hepatitis in infected inmates. Phylogenetic analysis of the partial 5′-UTR of the GBV-C/HGV genome from 16 seropositive individuals, which delineated their grouping within genotype 2, also revealed a close genetic relationship between two sets of sequences from 4 drug addicts, 3 of whom admitted to sharing needles while imprisoned. ***J. Med. Virol. 56:246–252, 1998.*** © 1998 Wiley-Liss, Inc.

KEY WORDS: viral hepatitis; RT-PCR; 5′-UTR

INTRODUCTION

Two independent groups of researchers recently discovered a novel member of the *Flaviviridae* family that was designated as GB virus C (GBV-C) [Simons et al., 1995; Leary et al., 1996], or hepatitis G virus (HGV) [Linnen et al., 1996]. GBV-C and HGV possess 85% and 95% identity at the nucleotide and amino-acid level, respectively, and they represent different isolates of the same blood-borne viral agent that resembles hepatitis C virus (HCV) in its genomic organization [reviewed in Simons et al., 1996]. Based on sequence analysis of 5′-ends of various isolates, GBV-C has been provisionally classified as genotype 1 and is more closely related to other West African isolates, whereas HGV has been classified as genotype 2a and bears a closer relationship to U.S. and European isolates [Muerhoff et al., 1996, 1997].

Infections with the virus have been found worldwide, not only in subjects with frequent parenteral exposure, such as intravenous drug users, and hemophilia and hemodialysis patients, but also in the general population; in fact, GBV-C/HGV was shown to be more prevalent in the U.S. population than HCV [Alter, 1996]. The circumstances that allow the virus to establish persistent infections at high prevalence rates, and yet in the absence of clinical manifestations of liver disease, remain unknown.

Detection of GBV-C/HGV-infected individuals currently relies on the measurement of GBV-C RNA with RT-PCR assays [reviewed in Simons et al., 1996]. Until recently, immunoreactive epitopes of GBV-C/HGV that could be used to develop a diagnostically useful serological test had not been identified; at present, antibodies to the putative second-envelope glycoprotein of the virus, which occur after the loss of GBV-C RNA in the

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serum and can persist for years after recovery from GBV-C/HGV infection, are thought to provide a measure of past exposure to the virus [Pilot-Matias et al., 1996; Tacke et al., 1997a].

In this study, samples from imprisoned Greek intravenous drug users were screened for viral hepatitis infection markers in order to investigate the prevalence and potential associations between infection with GBV-C/HGV and risk factors for seropositivity as well as with surrogate markers for possible liver damage. Nucleotide sequences from the partial 5′-UTR of the GBV-C/HGV genome of seropositive individuals were also isolated for phylogenetic analysis.

MATERIALS AND METHODS

Human Material

Sera from 106 male Greek intravenous drug users (mean age, 37 (SD, 9) years; range, 22–66 years), incarcerated at Agios Stefanos prison in Patras, Greece, were collected in March 1995, tested for alanine aminotransferase (ALT) levels, and stored at −70°C until use. The samples were screened retrospectively for GBV-C RNA, anti-GBV-C E2, HCV RNA, and anti-HCV, as well as for markers of HBV, HDV, and HIV infection. Each drug addict provided a blood sample after having completed a confidential questionnaire, which included sections on demography, legal condition, psychological state, and drug use and injecting behavior in general and during incarceration. The study was approved by the Ethics Committee of the Ministry of Justice, and all participants gave informed consent.

Determination of GBV-C RNA by RT-PCR

Extraction of RNA. Total RNA was extracted from 50 μl of serum with TRIZOL LS Reagent (Gibco BRL, Grand Island, NY), essentially according to the manufacturer's instructions. Nucleic acids were precipitated by ethanol in the presence of 10 μg of glycogen, resuspended in 30 μl of DEPC (diethylpyrocarbonate)-treated distilled water, and denatured by heating at 70°C for 10 minutes before proceeding to first-strand cDNA synthesis.

RT-PCR. Ten microliters of extracted RNA were converted to cDNA in a 20-μl reaction, using the GeneAmp RNA PCR Kit from Perkin Elmer (Roche Molecular Systems, Inc., Branchburg, NJ), basically according to the manufacturer's protocol. To allow for the extension of the random hexameric primers by MuLV Reverse Transcriptase (Perkin Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), reactions were initially incubated at room temperature for 10 minutes; after a subsequent incubation period of 90 minutes at 42°C, reverse transcription was stopped by heating at 99°C for 5 minutes.

Half of the reverse-transcribed cDNA was subjected to hot-start PCR in a total volume of 50 μl with primers (sequencing 5′-CGG CCA AAA GGT GGT GGA TG-3′ for the forward, and 5′-CGA CGA GCC TGA CGT CGG G-3′ for the reverse) that were deduced from the 5′-untranslated region (5′-NCR or 5′-UTR) of the GBV-C/HGV genome [Linnen et al., 1996]. All reagents used in the PCR were kindly provided by Boehringer Mannheim GmbH (Mannheim, Germany). The composition of the PCR mixture was 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3, 1.5 mM $MgCl_2$, 1 × PCR digoxigenin labeling mix (200 μM each of dATP, dCTP, and dGTP, 190 μM dTTP, and 10 μM digoxigenin-11-dUTP), 2.5 μM 5′-NCR primer mix, and 1.25 U of *Taq* DNA polymerase). PCR was performed for 45 cycles (consisting of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, and extension for 1 minute at 72°C), followed by an extension cycle at 72°C for 7 minutes to ensure completion of the reaction. Reaction conditions were adapted to the GeneAmp PCR System 2400 Thermal Cycler (Perkin Elmer, Foster City, CA). The expected size of the target sequence was 186 bp. To avoid cross-contamination, PCR was performed under the stringent conditions recommended by Kwok and Higuchi [1989]. One negative and two positive controls of low and high titer (Boehringer Mannheim GmbH), correspondingly, were used for every 10 samples; additionally, two negative controls (with DEPC-treated distilled water instead of RNA and cDNA, respectively) were also included in each run to monitor each stage of the procedure and to ensure the specificity of amplification products.

Detection of amplification products. The detection of GBV-C/HGV-specific, digoxigenin-labeled DNA was carried out in streptavidin-coated microtiter plates by using the PCR Elisa DIG Detection kit (Boehringer Mannheim GmbH), according to the manufacturer's instructions. A serum sample was deemed positive if its absorbance was greater than three times the absorbance of a GBV-C-negative serum sample. To exclude false-positive signals, all samples that had been found GBV-C RNA-positive in the initial screening were prepared and assayed a second time.

DNA Sequencing and Phylogenetic Analysis

Prepared cDNA samples from all drug addicts shown to be infected with GBV-C/HGV were subjected to PCR using primers S1, GBVCE1wb2, and 4R in a semi-nested fashion, as previously described [Muerhoff et al., 1996]. The amplified 448-bp fragments from the 5′-UTR of the GBV-C/HGV genome were purified using the GeniePrep kit (Ambion, Inc., Austin, TX) and sequenced directly for both strands on an ABI PRISM 377 automated DNA sequencer (Applied Biosystems), using a dye terminator cycle sequencing kit (Perkin Elmer).

Nucleotide sequences thus obtained were aligned with the corresponding genomic region of previously reported sequences, using the CLUSTAL V program [Higgins and Sharp, 1989]. Pairwise distance matrices were calculated by the algorithm described by Kimura [1980], and dendrograms were constructed with the DNADIST and NEIGHBOR programs of the PHYLIP package, version 3.5 [Felsenstein, 1993] by the neighbor-joining method [Saitou and Nei, 1987]. SEQBOOT and CONSENSE were used for bootstrap analysis (100 replicates) in order to estimate the reliability of the

produced phylogenetic trees, which were visualized and edited graphically with TREEVIEW [Page, 1996].

The GenBank accession numbers of the reference sequences used in the phylogenetic analysis, according to their provisional genotypic classification, are as follows: genotype 1, U36380, U59540, U59542, U59547, and U59551; genotype 2a, U44402, U59519, U59522, U59524, U59526, U59527, and U59528; genotype 2b, U59529, U59530, and U59531; and genotype 3, U59538 and U59539.

Detection of Anti-GBV-C E2

The qualitative determination of IgG antibodies to the GBV-C/HGV E2-antigen in serum samples was performed using the μPLATE Anti-HGenv kit (Boehringer Mannheim GmbH), in accordance with the manufacturer's suggested protocol. Results were determined by using a cutoff value given by the formula

$$A_{cutoff} = 0.2 \times A_{pos} + A_{neg},$$

where A_{pos} and A_{neg} represented the absolute values of absorbance of the positive and negative control, respectively [Tacke et al., 1997a].

Markers of HCV, HBV, HDV, and HIV Infection

Serum samples were tested for HCV RNA with a commercially available RT-PCR assay (Amplicor, Roche Diagnostic Systems, Inc., Branchburg, NJ). Antibodies to HCV (anti-HCV) were detected in serum by a third-generation enzyme-linked immunosorbent assay (Ortho Diagnostic Systems, Tokyo, Japan). Samples were also tested for hepatitis B core antibody (anti-HBc) using the Corzyme Diagnostic Kit, and for hepatitis B surface antigen (HBsAg) using the Auszyme Monoclonal Diagnostic Kit (both from Abbott Laboratories, Abbott Park, IL). Samples found reactive by anti-HBc and HBsAg testing were screened for antibodies to the hepatitis delta virus (anti-HDV), using the Anti-Delta EIA Diagnostic Kit (Abbott Laboratories). HIV-1 antibody testing (anti-HIV1) was performed using enzyme-linked immunoassays, with confirmation by Western blot (Cambridge Biotech Corp., Worcester, MA).

Statistical Analyses

Relationships between GBV-C/HGV status and questionnaire-derived variables or variables indicating HCV status and ALT levels were examined by chi-square test, t-test, or Wilcoxon rank-sum test, as appropriate. Multiple logistic regression was used to determine whether GBV-C/HGV serostatus was independently associated with ALT levels. A P value of 0.05 or less was considered statistically significant.

RESULTS

Prevalence of GBV-C/HGV, HCV, HBV, HDV, and HIV Infection Markers

The outcome of the screening of sera from Greek intravenous drug users for viral hepatitis markers is summarized in Table I. GBV-C RNA and anti-GBV-C E2 were detected in 34 (32.1%) and 49 (46.2%) of the tested samples, whereas HCV RNA and anti-HCV were detected in 68 (64.2%) and 82 (77.4%) of the tested samples, respectively. Among the 106 drug users, 59 (55.7%) were positive for the hepatitis B core antibody, and 9 (8.5%) were positive for the hepatitis B surface antigen. Of the 9 samples that had been found reactive by both anti-HBc and HBsAg testing, only 3 were determined to be anti-HDV positive. HIV-1 antibody was not detected in any sample.

TABLE I. Prevalence and 95% Confidence Intervals (CI) for Viral Hepatitis Markers Among Imprisoned Greek Intravenous Drug Users (N = 106)

Viral hepatitis marker	Positive samples	Prevalence (%)	95% CI
GBV-C RNA	34	32.1	(23.3, 41.9)
Anti-GBV-C E2	49	46.2	(36.5, 56.2)
HCV RNA	68	64.2	(54.3, 73.2)
Anti-HCV	82	77.4	(68.2, 84.9)
Anti-HBc	59	55.7	(45.7, 65.3)
HBsAg	9	8.5	(4.0, 15.5)
Anti-HDV	3	2.8	(0.5, 8.0)

Univariate Associations Between Markers of GBV-C/HGV and HCV Infection With Potential Risk Factors for Seropositivity

Characteristics of the tested incarcerated injecting drug users according to their GBV-C/HGV serostatus are shown in Table II. A significant association of active GBV-C/HGV infection was found with age, with younger drug abusers exhibiting an increased risk of GBV-C/HGV-seropositivity ($P = 0.006$). GBV-C RNA-positive individuals were also likely to be coinfected with HCV ($P = 0.024$). Univariate analysis identified a significant association between GBV-C RNA seropositivity and higher alanine aminotransferase levels in injecting drug users ($P < 0.001$).

Neither GBV-C RNA (Fig. 1) nor anti-GBV-C E2 (data not shown) was determined to be associated in a statistically significant manner with any of the other potential risk factors described in the questionnaire. In contrast, HCV-RNA correlated significantly with several such variables; i.e., significant univariate associations were found between HCV-seropositivity and the duration of intravenous use ($P = 0.042$), the sharing of needles ($P = 0.023$), and the duration of incarceration ($P = 0.035$). Noteworthy is the observation that many drug users continued not only to inject, but also to share needles in prison.

To investigate further the association between GBV-C RNA positivity and higher ALTs, a multiple logistic regression model was applied. GBV-C RNA seropositive status was therefore found to be independently associated with raised ALT levels ($P = 0.039$), even after adjusting for HCV-RNA status and age, and after excluding the 9 individuals who had tested positive for the hepatitis B surface antigen (Table III). In fact, aminotransferase levels were found to be greater

TABLE II. Characteristics of Imprisoned Greek Intravenous Drug Users, According to GBV-C-RNA Serostatus

Characteristics	GBV-C RNA Positive (N = 34)	GBV-C RNA Negative (N = 72)	*P*
Age (years; mean (SD))	33.5 (7.6)	38.5 (9.2)	0.006
ALT (IU/l; median (25th, 75th))[a]	103 (38, 219)	36 (22, 82)	<0.001
Positive for, N (%)			
Anti-GBV-C E2	8 (23.5%)	41 (56.9%)	0.001
HCV RNA	27 (79.4%)	41 (56.9%)	0.024
Anti-HCV	29 (85.3%)	53 (73.6%)	NS[b]
Anti-HBc	18 (52.9%)	41 (56.9%)	NS
HBsAg	2 (5.9%)	7 (9.7%)	NS
Anti-HDV	1 (2.9%)	2 (2.8%)	NS

[a]ALT, upper limit of normal, 35 IU/l.
[b]NS, not significant.

than six times the upper limit of normal (>210 IU/l) in 29.6% of GBV-C-RNA- and HCV-RNA-infected individuals, in comparison to 0% of individuals infected with GBV-C-RNA alone, 5.4% with HCV-RNA alone, and 3.6% with neither ($P = 0.006$).

Molecular Characterization of GBV-C/HGV-Seropositive Samples

All GBV-C-RNA-positive samples were subjected to an additional set of amplification reactions in order to be analyzed phylogenetically. As shown in Figure 2, phylogenetic analysis of unique 5′-UTR sequences obtained from 16 GBV-C/HGV-seropositive Greek intravenous drug users delineated their grouping within genotype 2, along with reference isolates derived from other parts of Europe and the U.S. [Muerhoff et al., 1996]. More specifically, 15 isolated sequences were determined to belong to the 2a subgroup, while only one, IVDU98-GR, was determined to belong to the 2b subgroup.

IVDU87-GR appeared to be an outgroup of 2a sequences; nonetheless, it was assigned to subgroup 2a, since the bootstrap values associated with the clustering of IVDU87-GR with 2a sequences were significant (75%) (Fig. 2). Omission of this sequence from analysis increased the degree of confidence of the grouping of 2a sequences from 75% to 88% (data not shown).

Despite the fact that the 5′-UTR of the GBV-C/HGV genome is relatively well-conserved among isolates, no identical sequences were found. However, two sets of sequences, IVDU86-GR with IVDU24-GR (99 replicates out of 100) and IVDU44-GR with IVDU14-GR (84 replicates out of 100), were closely related (Fig. 2).

DISCUSSION

In addition to the expected high rates of viral hepatitis C infection markers (64.2% for HCV RNA, 77.4% for anti-HCV, and 79.3% for the combination of the two markers), the current study revealed a lower, but still high GBV-C/HGV seroprevalence rate (32.1% for GBV-C RNA, 46.2% for anti-GBV-C E2, and 70.8% for the combination of these two infection markers) among imprisoned intravenous drug users in Greece.

The observed active GBV-C/HGV infection rate among the 106 tested injecting drug users (32.1%) is in accordance with the rates previously reported in other countries for this population group [Diamantis et al., 1997; Tacke et al., 1997a; Schlueter et al., 1996; Linnen et al., 1996; Stark et al., 1996; Hadziyannis et al., 1995]. A similar study carried out solely with primer pairs derived from the NS3 helicase region of the viral genome detected a lower percentage of GBV-C RNA (24%) among Japanese patients with HCV-associated chronic liver disease who had abused drugs intravenously [Aikawa et al., 1996]. This reported lower prevalence rate may reflect a lower degree of conservation of the nonstructural domains of GBV-C/HGV in comparison to its 5′-UTR, especially since coinfection with GBV-C/HGV does not appear to influence the clinical or virologic course of HCV infection [Tanaka et al., 1996].

Indeed, the existence mainly of covariant substitutions in the 5′-UTR within the protective form of a loop structure and the low amount of variability between GBV-C/HGV isolates within this region in general suggest that the 5′-UTR contains secondary structures critical for viral replication, packaging, and translation initiation [Erker et al., 1996]. However, since the nucleotide divergence within the primer target region is still unknown, undertaking an amplification in parallel with a primer pair from a different region of the GBV-C/HGV genome, such as the NS3 or NS5 domain, could have enhanced the sensitivity of the RT-PCR assay used. Still, the good conservation of the 5′-UTR of the GBV-C/HGV genome that is driven by strong evolutionary constraints [Erker et al., 1996] renders it appropriate for use in detection of viremia and molecular characterization of seropositive samples [reviewed in Simons et al., 1996].

The high prevalence rate of antibodies to the E2 glycoprotein of GBV-C/HGV (46.2%, Table I) suggests that in comparison to drug abusers with active infection, an even larger percentage of individuals had been exposed to GBV-C/HGV in the past. This frequency is comparable to those obtained by Tacke et al. [1997a,b], who reported rates of 41% and 54% among injecting drug addicts from Germany and Spain, respectively. Pilot-Matias et al. [1996] and Dille et al. [1997] reported even larger percentages of anti-GBV-C E2-positive individuals (72.5% and 85.2%, respectively) among injecting drug users, possibly due to differences in the assay formats or demographic variables. Testing for anti-GBV-C E2 extends the ability of RT-PCR to define the epidemiology of the virus, since it constitutes a marker of clearance of GBV-C/HGV infection [Tacke et al., 1997a]. Further studies will establish whether this antibody also constitutes a marker of immunity.

GBV-C RNA and anti-GBV-C E2 may be detected simultaneously only for a limited time interval [Tacke et al., 1997a]. In accordance with this expectation, only 8 of the 34 GBV-C-RNA-positive drug users were determined to be anti-GBV-C E2-positive. Since most

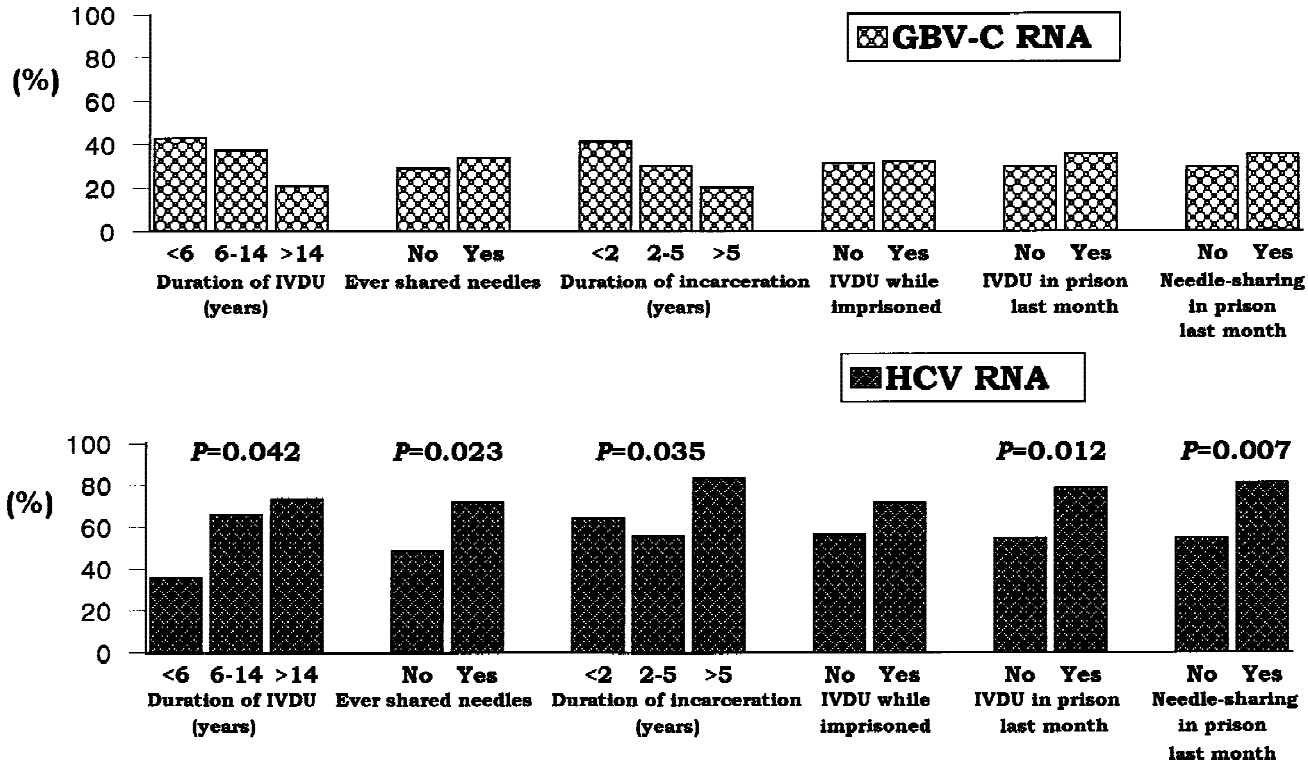

Fig. 1. Univariate associations between GBV-C- and HCV-seropositivity and potential risk factors among incarcerated intravenous drug users in Greece. *P* values are mentioned for statistically significant associations only. IVDU, intravenous drug use.

TABLE III. Association of GBV-C-RNA Status of Imprisoned Greek Intravenous Drug Users With ALT Levels, Adjusting for Age and HCV RNA (Multiple Logistic Regression Model)

Variables	Odds ratio	95% CI	*P*
Age	0.95	(0.89, 1.02)	0.134
HCV RNA (+ vs. –)	2.20	(0.63, 7.62)	0.211
ALT (log values)	1.86	(1.03, 3.37)	0.039

GBV-C/HGV-seropositive individuals were anti-GBV-C E2- negative (Table II), perhaps they had been recently infected with GBV-C/HGV. The lower seroprevalence rate of GBV-C/HGV with respect to HCV (Table I), and the younger age of GBV-C-RNA-positive individuals (Table II), an association which is in accordance with the findings of Thomas et al. [1997], also point to the conclusion that in this group of drug addicts, GBV-C/HGV was introduced more recently than HCV.

The association of relatively recent GBV-C/HGV infection with higher alanine aminotransferase levels among these imprisoned intravenous drug users ($P < 0.001$, Table II) was surprising. Tacke et al. [1997a] also found increased serum ALT concentrations in 25% of drug users positive for GBV-C RNA vs. 18.8% in those who were GBV-C-RNA- and anti-E2-negative. In contrast, neither Thomas et al. [1997] nor Zehender et al. [1998] reported a significant difference in mean serum ALT levels between GBV-C-RNA-positive and GBV-C-RNA-negative patients. Diamantis et al. [1997], in particular, stated that the number of participants of their studies with elevated ALT levels did not differ significantly whether patients were infected with HCV alone or coinfected with GBV-C/HGV.

In our study, GBV-C/HGV-seropositive drug users were more prone to have elevated ALTs, irrespective of their age and of their being coinfected with HCV ($P = 0.039$, Table III), probably because they had been infected recently in prison and were suffering from acute subclinical hepatitis due to GBV-C/HGV. The much larger percentage of drug addicts who were coinfected with GBV-C/HGV and HCV (29.6%) and whose ALTs levels were greater than six times the upper limit of normal (>210 IU/l), in comparison to individuals infected with GBV-C/HGV alone (0%), HCV alone (5.4%), and with neither (3.6%) ($P = 0.006$), further enhances the possibility that an acute infection was ongoing at the time of testing.

Furthermore, phylogenetic analysis of 5′-UTR sequences from 16 GBV-C/HGV-seropositive drug abusers, which delineated their grouping within genotype 2 (Fig. 2) along with isolates derived from other parts of Europe and the U.S. [Muerhoff et al., 1996], also revealed a close genetic relationship between two sets of sequences from 4 drug addicts (Fig. 2); 3 of these individuals admitted to abusing drugs intravenously and sharing needles while imprisoned. Hence, GBV-C/HGV seems to have spread readily through needle-sharing

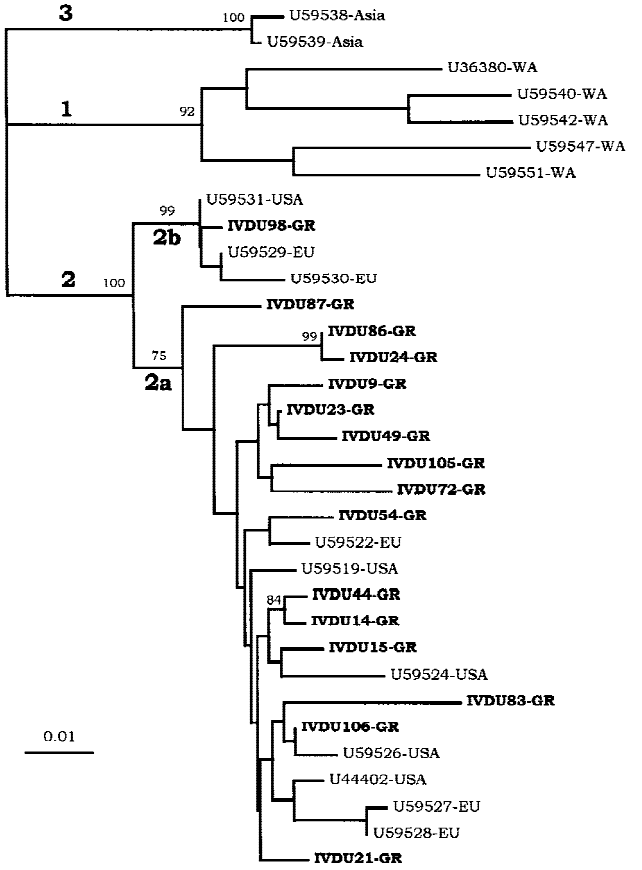

Fig. 2. Phylogenetic tree based on comparison of 5′-UTR sequences from 16 Greek intravenous drug users (IVDUs) with 17 GBV-C/HGV representative reference sequences from around the world. Numbers at nodes of the tree indicate bootstrap values obtained from 100 replicas. Non-Greek isolates are referred to by their GenBank accession number and their origin. GR, Greece; EU, Europe; WA, West Africa.

among imprisoned drug users, while inducing an increase in serum ALT levels of infected inmates.

In conclusion, these data corroborate earlier findings which clearly indicate that parenteral exposure by intravenous drug abuse constitutes a vehicle for the spread of GBV-C/HGV [Aikawa et al., 1996]. Given their common route of transmission via blood and blood products, it is not surprising that coinfections occur with HCV and GBV-C/HGV. Prevention of HCV transmission will also stop the circulation of GBV-C/HGV.

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